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1-PHENYL-3-TRIMETHYLAMINOPROPYL CARBODIIMIDE: A NEW INHIBITOR OF THYMIDYLATE SYNTHASE

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Thymidylate synthase (EC 2.1.1.45) from methotrexate-resistant *Lactobacillus casei* was inactivated by 1-phenyl-3-trimethylaminopropyl carbodiimide (PTC), 1-phenyl-3-dimethyl aminopropyl carbodiimide (PDC), and 1-ethyl-3-dimethyl aminopropyl carbodiimide (EDC). In the presence of excess PTC, the inactivation followed pseudo-first order kinetics; the second order rate constant was approximately 200 $M^{-1}min^{-1}$ at 30°C. The rate of inactivation by PTC was faster than that by either PDC or EDC. Concentrations of the substrate dUMP greater than 0.15 mM, or of the product dTMP greater than 1.6 mM completely protected the enzyme from inactivation by PTC, but 10 mM dUrd provided very little protection. The rate of inactivation of EDC was reduced by only 40% in the presence of 50 mM dUMP. Nucleophiles (sulfanilic acid, glycine methyl ester, or glycine ethyl ester) had no effect on the rate of inactivation by PTC.

The complete inactivation of thymidylate synthase by PTC was accompanied by the incorporation of approximately 2 mols of ^{14}C-PTC per mol of enzyme. Although carbodiimides normally modify carboxyl groups in proteins, results from sulfhydryl group titrations and from reversible modification of sulfhydryl groups by methyl methanethiosulfonate suggest that two of the four cysteine residues of thymidylate synthase were modified by PTC.

KEY WORDS: Thymidylate synthase, inhibition, carbodiimide synthesis.

INTRODUCTION

Thymidylate synthase (EC 2.1.1.45) catalyzes the reductive methylation of 2′-deoxyuridylate by 5,10-methylene-5,6,7,8-tetrahydrofolate to produce thymidylate and 7,8-dihydrofolate. Because it is essential in the *de novo* synthesis of thymidylate, the methanism of thymidylate synthase has been extensively investigated. The first indication that a carboxyl group might be catalytically important was the observation[1] that thymidylate synthase was completely inactivated by removing a single C-terminal valine from one of the two subunits with carboxypeptidase-A. A later investigation showed that the C-terminal valine was involved in cofactor binding.[2]

Since carbodiimides have been used for the specific modification of carboxyl groups in proteins,[3] we investigated the reaction of thymidylate synthase with three water-soluble carbodiimides, two of which were synthesized in our laboratory. All of the

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Abbreviations. dTMP, thymidylate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); dUMP, deoxyuridylate; EDC, 1-ethyl-3-dimethyl aminopropyl carbodiimide; GEE, glycine ethyl ester; GME, glycine methyl ester; MES, 2-(N-morpholino)ethane-sulfonic acid; MMTS, methyl methanethiosulfonate; PDC, 1-phenyl-3-dimethylaminopropyl carbodiimide; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); PMB, *p*-mercuribenzoic acid; PTC, 1-phenyl-3-trimethylaminopropyl carbodiimide; TCA, trichloroacetic acid; TS, thymidylate synthase.

carbodiimides tested inhibited thymidylate synthase, but the inactivation was not affected by the presence of nucleophiles and appeared to be due to reaction with catalytically-important sulfhydryl groups rather than carboxyl groups in the enzyme.

MATERIALS AND METHODS

Materials

Chloroform-d_1, N,N-dimethylaminopropaneamine, dimethyl sulfoxide-d_6, *p*-toluenesulfonyl chloride, phenylisothiocyanate, and tetramethylsilane were obtained from Aldrich Chemical Company (Milwaukee, WI). A Protein Assay Kit was from Bio-Rad Laboratories (Richmond, CA). Methyl iodide was from Eastman Kodak (Rochester, NY). Sephadex G-25 (super fine) was from Pharmacia Fine Chemicals (Piscataway, NJ). Pyridine (sequanal grade) and triethylamine (sequanal grade), were from Pierce Chemical Company (Rockford, IL). Triton X-100 (scintillation grade) was from Research Products International (Mount Prospect, IL). Deoxyuridine, the disodium salt of 2′-deoxyuridine-5′-monophosphate, 5,5′-dithiobis-(2-nitrobenzoic acid), folic acid, glycine methyl ester, 2-mercaptoethanol, ^{14}C-methyl iodide (42.8 mC_i/mmol), methyl methanethiosulfonate, 2-(morpholino)ethane sulfonic acid, *p*-chloromercuribenzoic acid, piperazine-N,N′-bis(2-ethanesulfonic acid), sulfanilic acid, thymidine monophosphate, and trichloroacetic acid were from Sigma Chemical Company (St. Louis, MO). All other chemicals and reagents used were obtained commercially and were of reagent grade or better.

(±)-Tetrahydrofolic acid was prepared by the catalytic hydrogenation of folic acid in acetic acid.[4] The product was lyophilized, sealed under argon in serum bottles[5] and stored at −50°C. A stock solution of (±)-5,10-methylenetetrahydrofolate was prepared by dissolving 6 mg of (±)-tetrahydrofolic acid in 5 ml of a solution which contained 0.5 ml of 0.5 M sodium bicarbonate (pH 8.1), 1.25 ml of 0.268 M formaldehyde, 1.25 ml of 1 M 2-mercaptoethanol, and 2 ml of distilled water.

Thymidylate synthase was purified from methotrexate-resistant *Lactobacillus casei* cells by the procedure of Lyon *et al.*[6] The enzyme was activated by dialysis at 5°C for 18–24 h against 20 mM potassium phosphate buffer, pH 6.8, containing 25 mM 2-mercaptoethanol. 2-Mercaptoethanol was removed immediately before use by adsorbing the enzyme on CM-Sephadex,[7] washing with 50 mM MES buffer, pH 6.0 and eluting the protein from the gel with the same buffer containing 0.75 M KCl.

Methods

Enzyme assay Thymidylate synthase was assayed at 30°C with a Gilford Model 250 spectrophotometer (Gilford Instruments Co., Oberlin, OH) as previously described.[8] Standard assays were initiated by adding enzyme to otherwise complete reaction mixtures. However, when the enzyme had been inactivated with MMTS or PMB, it was added to assay mixtures containing 100 mM 2-mercaptoethanol and lacking dUMP. After 5 min, dUMP was added to initiate the reaction. Protein concentrations were determined either spectrophotometrically[6] or by the dye-binding assay of Bradford.[9]

Protection of sulfhydryl groups The sulhydryl groups of dethiolated thymidylate synthase were blocked by treatment with a 10- to 15-fold molar excess of MMTS or

PMB. When the enzyme activity was less than 2% of the initial activity, excess reagents were removed by gel filtration on a Sephadex G-25 column (2 × 20 cm or 1 × 18 cm) which was equilibrated with 50 mM MES buffer, pH 6.0.

Titration of sulfhydryl groups The concentration of free sulfhydryl groups was determined by titration with DTNB[10] in the presence of urea. A 30- to 40-fold molar excess of DTNB was added to the enzyme from a stock solution containing 10 mM DTNB in 10 mM Na_2CO_3 and 0.1 M sodium phosphate at pH 7.0. After 5 min incubation, the absorbance at 412 nm was measured against a blank containing DTNB in buffer. Solid urea was then added to a final concentration of 5.7–7.3 M and the A_{412} was measured after 5 min. The sulfhydryl concentration was determined from the molar absorptivity of 13,600 $M^{-1}cm^{-1}$ for nitromercaptobenzoate.[10]

Liquid scintillation counting Radioactive protein samples were purified by Sephadex G-25 chromatography. Aliquots (up to 1 ml) were added to 5 ml of toluene/Triton X-100 (2/1, v/v) containing 4% (w/v) OMNIFLUOR. Radioactivity was measured in a Searle Model Delta 300 liquid scintillation spectrophotometer (Searle Analytic Inc., Des Plaines, IL). Alternatively, protein samples were precipitated in 12.5% TCA (final concentration). The precipitate was filtered on a Whatman GF/B glass microfiber filter (Whatman Lab Sales Inc., Hillsboro, OR), washed successively with 5 ml 12.5% TCA, 5 ml 1 N HCl, 5 ml ethanol, and three times with 5 ml acetone. The filters were dried under a heat lamp and added to the scintillation cocktail; radioactivity was measured as described above.

Synthesis of 1-phenyl-3-(3-trimethyl-aminopropyl) carbodiimide Carbodiimides were synthesized using the method of Sheehan *et al.*[11] with minor modifications. 1-Phenyl-3(3-dimethylaminopropyl) thiourea was prepared by slowly adding over a 1 h period an ether solution of N,N-dimethylaminopropaneamine (0.05 mols) to an equal molar amount of phenylisothiocyanate in ether that was kept in an ice bath and vigorously stirred. The reaction mixture was then stirred for an additional 5 h at room temperature. The resulting crystalline thiourea was collected by filtration, dissolved in methylene chloride and triethylamine, placed in an ice bath, and a 2-fold molar excess of *p*-toluene-sulfonylchloride in methylene chloride was slowly added with vigorous stirring. The mixture was refluxed for 4 h and extracted 3 times with 40% potassium carbonate. The organic phase was concentrated using a Büchi Rotavapor R110 (Brinkmann Instruments, Inc., Westbury, NY) and the residual oil extracted 3 times with ether. After the ether was evaporated, the residue was distilled to give about 0.02 mol (47% yield) of a colorless, oily liquid which was 1-phenyl-3-(3-dimethyl-amino-propyl) carbodiimide(PDC) (b.p. 89°C, 0.35 mm). Dry HCl gas was bubbled through a solution of PDC in ether to make the hydrochloride. The resulting product was collected by filtration and recrystallized 2 times from acetone/ether (m.p. 129–131°C). 1-Phenyl-3-(3-trimethylaminopropyl) carbodiimide(PTC) was synthesized by slowly adding a 2-fold molar excess of methyl iodide in ether to a PDC solution in ether. The resulting precipitate was collected by filtration and recrystallized 2 times from acetone/ether (m.p. 158–159.5°C) to obtain 2.5 g (about 36% yield) of PTC.

The identities of the final product (PTC) and the intermediate (PDC) were confirmed by ^{1}H and ^{13}C-nmr spectra (Bruker AM-400 spectrometer). The molar absorptivity of PTC in 50 mM MES buffer, pH 6, was 18,500 $M^{-1}cm^{-1}$ at 228 nm. (HP-8451A diode array spectrophotometer, Hewlett-Packard, Palo Alto, CA), and this value was used to calculate PTC concentrations.

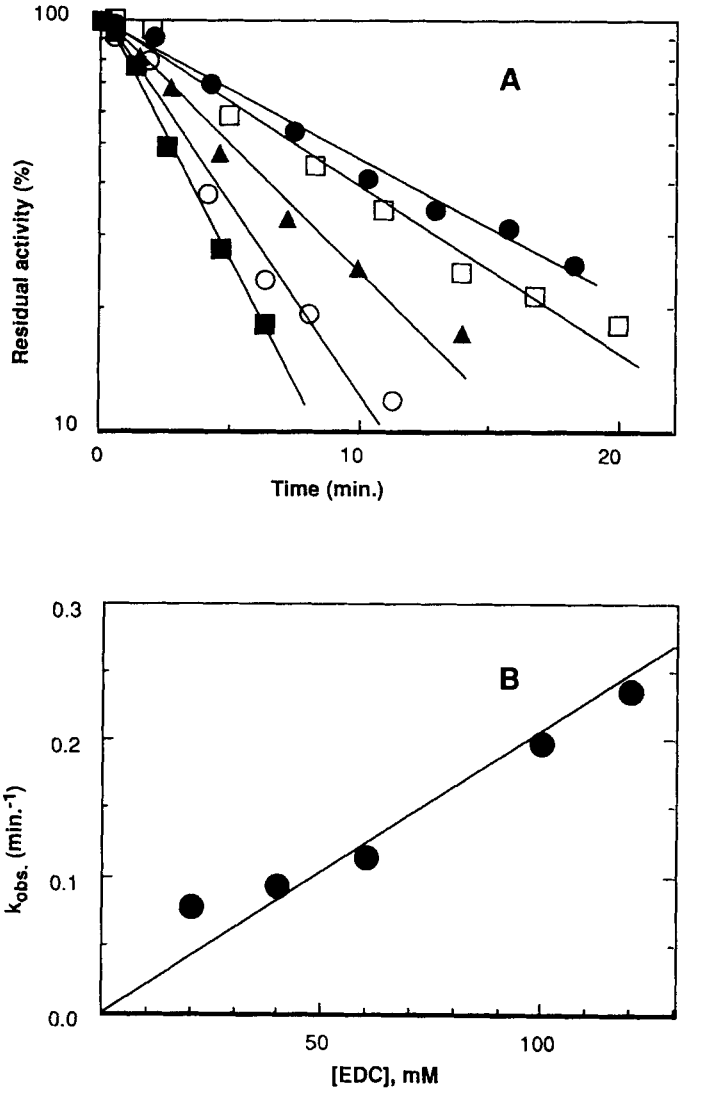

FIGURE 1 Inactivation of thymidylate synthase by EDC. A. Dethiolated 8.6 μM TS was incubated at 30°C in 50 mM MES buffer, pH 6.0 containing EDC at the following concentrations: 20 mM (●), 40 mM (□), 60 mM (▲), 100 mM(○), 120 mM (■). Aliquots (20 μl) were withdrawn periodically and assayed for enzymatic activity. The activity is plotted against the incubation time and is expressed as a percent of the activity of a reaction mixture lacking EDC. B. The second order rate constant was determined from the slope of the replot of the pseudo first order rate constants (k_{obs}) vs the corresponding EDC concentrations.

RESULTS

The initial observation that TS was inactivated by EDC (Aull, unpublished results) was extended by examining the kinetics of the inactivation, and the results are shown in Figure 1. The linear plots in Figure 1A indicate that the inactivation was first-order

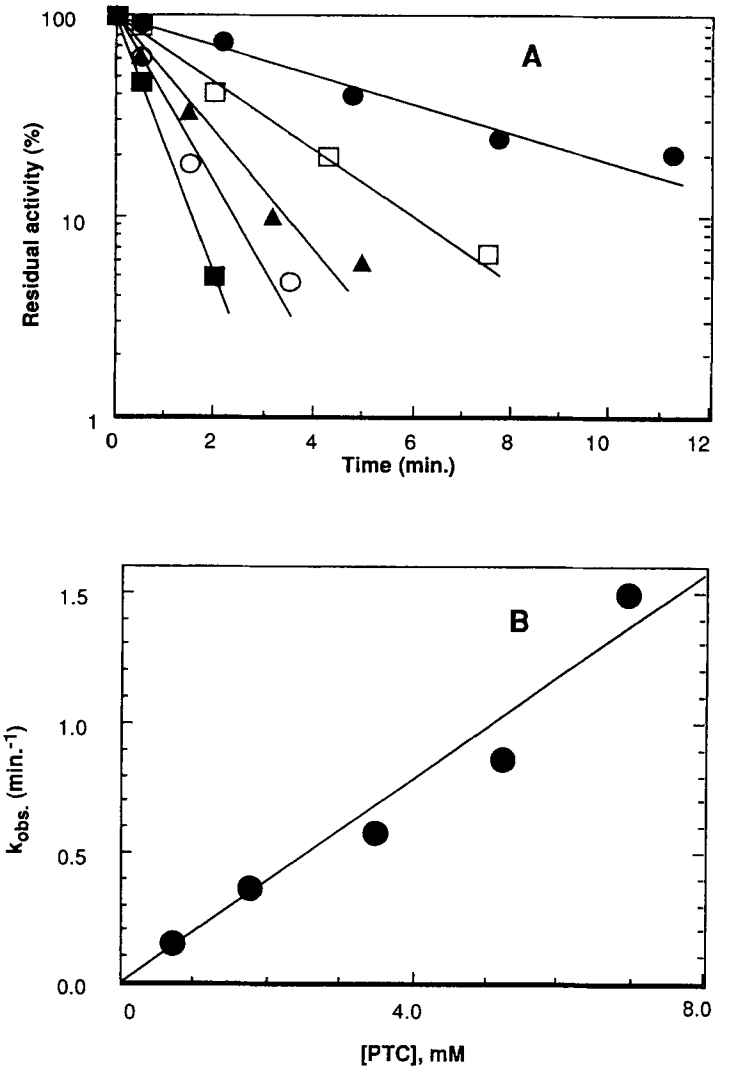

FIGURE 2 Inactivation of thymidylate synthase by PTC. A. Dethiolated 10 μM TS was incubated at 30°C in 50 mM MES buffer, pH 6.0 containing PTC at the following concentrations: 0.69 mM (●), 1.74 mM (□), 3.47 mM (▲), 5.21 mM (○), and 6.9 mM (■). Aliquots (20 μl) were withdrawn periodically and assayed for enzymatic activity. The activity is plotted against the incubation time and is expressed as a percent of the activity of a reaction mixture lacking PTC. B. The second order rate constant was determined from the slope of the replot of the pseudo first order rate constants (k_{obs}) vs the corresponding PTC concentrations.

with respect to enzyme concentration and that the rate of inactivation was a function of the carbodiimide concentration. A plot of the apparent first-order rate constants, obtained from the slopes of the lines, *versus* the corresponding concentrations of EDC (Figure 1B) was approximately linear, and a second-order rate constant of 2.1 $M^{-1}min^{-1}$ was calculated from the slope. The need for radioactive, water soluble

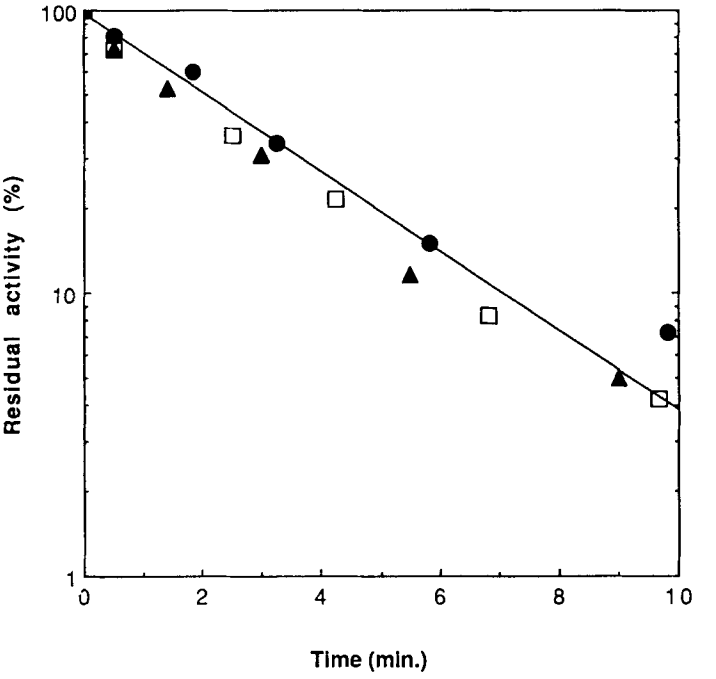

FIGURE 3 Effect of nucleophiles on thymidylate synthase inactivation by PTC. Enzyme (12 μM) was incubated with PTC (2.3 mM) at 30°C in 50 mM MES buffer, pH 6.0 in the absence of added nucleophiles (□), and in the presence of 30 mM sulfanilate (●) or 4 mM ethidium bromide (▲). Aliquots (20 μl) were withdrawn periodically and assayed for enzymatic activity.

carbodiimides for binding studies led to the synthesis of other carbodiimides, since radiolabeled EDC was not commercially available. PTC was synthesized and found to be a better inhibitor of TS than EDC (Figure 2). The second order rate constant of 200 $M^{-1}min^{-1}$ for PTC is about 100 times greater than that for EDC. PDC, an intermediate in the synthesis of PTC, also inactivated TS at a rate between those of PTC and EDC (data not shown). Thymidylate synthase was inactivated by PTC at the same rate and to the same extent in either the presence or the absence of nucleophiles (Figure 3). The failure of nucleophiles to enhance inactivation suggests that the loss of enzyme activity is caused by a reaction between the enzyme and PTC alone, in which nucleophiles are apparently not directly involved. Dialyzing these reaction mixtures overnight against 20 mM phosphate buffer, pH 6.8, containing 10 mM 2-mercaptoethanol did not regenerate any significant activity. PTC and the enzyme apparently interact to form a relatively stable product, since the inactivation was time dependent and was not readily reversible.

The enzyme was protected from inactivation by PTC when either dUMP or dTMP was included in the reaction mixtures (Figure 4), but deoxyuridine, which is not a substrate, did not provide appreciable protection even when present at a relatively high (10 mM) concentration. In contrast to the results with PTC, the rate of inactivation by 80 mM EDC was reduced by only 40% in the presence of high concentrations (50 mM) of dUMP (data not shown).

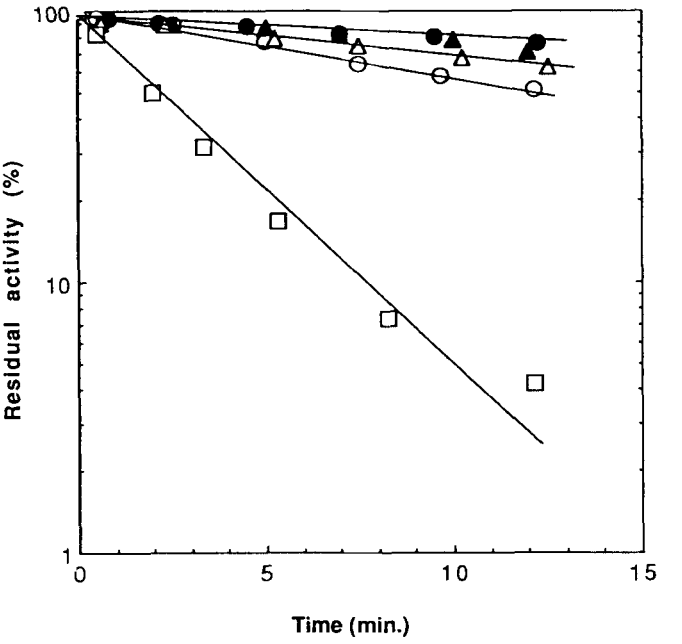

FIGURE 4 Effect of dUMP and dTMP on the inactivation by 2.5 mM PTC. Thymidylate synthase (16 μM) was incubated at 30°C in 50 mM MES buffer, pH 6.0 in the absence of nucleotide (□), and in the presence of 0.142 mM (●) or 0.029 mM (○) dUMP; or 1.6 mM (▲) or 0.321 mM (△) dTMP. Aliquots (20 μl) were withdrawn periodically and assayed for enzyme activity.

The inactivation by carbodiimides in the absence of nucleophiles and the partial protection provided by the substrate suggested that some functional groups other than carboxyl groups of TS had reacted. The most likely candidate was cysteine sulfhydryl groups, since thymidylate synthase is known to contain essential cysteine residues and carboniimides react with sulfhydryl compounds.[12] The sulfhydryl groups of thymidylate synthase were prevented from reacting with PTC by treating the enzyme with MMTS prior to exposure to PTC. MMTS reacted with approximately two of the four cysteines of thymidylate synthase and completely inactivated the enzyme. The activity can be completely regenerated by adding excess thiols such as 2-mercaptoethanol to the inactivated enzyme. Data in Figures 3 and 5 show that MMTS-treated TS was inactivated by PTC at a much slower rate than the untreated enzyme and that added nucleophiles had no effect on the rate of inactivation. Titrations with DTNB indicated that PTC-inactivated TS had 1.2 less titratable sulfhydryl group than the native enzyme (data not shown).

Thymidylate synthase was inactivated with ^{14}C-PTC in order to correlate the extent of inactivation with the extent of PTC incorporation (Table I). Approximately 2 mols of PTC were incorporated per mol of TS during the first 10–11 min, during which time the enzyme was almost completely inactivated. This rapid inactivation was followed by the slower incorporation of an additional 2 mols of PTC over the next 4 h. However, only 2 mols of PTC were incorporated into MMTS-treated enzyme after 4 h. These results also suggest that PTC reacts with sulfhydryl groups.

Carbodiimides have also been reported to react with hydroxyl groups of tyrosine

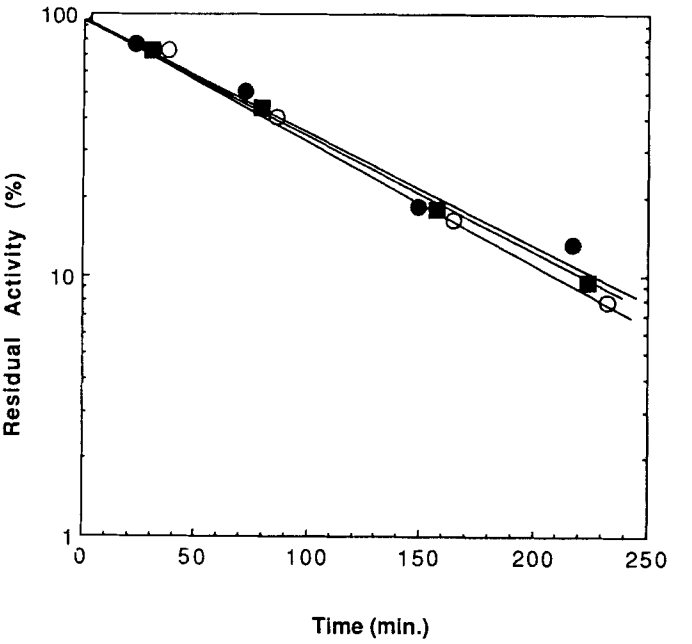

FIGURE 5 Effect of nucleophiles on the inactivation of MMTS-treated thymidylate synthase by PTC. Dethiolated enzyme was treated with a 10- to 15-fold molar excess of MMTS until > 98% of the enzyme activity was lost. Excess MMTS was removed by Sephadex G-25 gel filtration chromatography equilibrated in 50 mM MES buffer, pH 6.0. MMTS-treated enzyme (12.5 μM) was reacted with PTC (8.3 mM) in the absence of added nucleophiles (○), and presence of 14 mM sulfanilate (●) or 14 mM glycine methyl ester (■). Aliquots (20 μl) were withdrawn at the times shown and added to assay mixtures containing 100 mM 2-mercaptoethanol but lacking dUMP. dUMP was added after a 5 min incubation period to initiate the catalyzed reaction.

residues in proteins but the tyrosine residues can be regenerated by treatment with high concentrations of hydroxylamine.[13] Incubating PTC-treated TS with 0.67 M hydroxylamine for 4 h resulted in the recovery of some of the catalytic activity and the release of approximately one-half of the bound PTC whether the PTC treatment had

TABLE I
PTC Binding to Thymidylate Synthase

Enzyme pretreatment	Time[a] (min)	Activity[b] (%)	PTC[c] bound (nmols)	TS (nmols)	PTC/TS
none	10	5	24	18	1.3
none	11	2	30	14	2.1
none	11	5	51	25	2.0
none	240	0	113	32	3.5
none	240	0	69	18	3.8
MMTS[d]	240	15	82	38	2.2
MMTS[d]	240	15	28	14	2.0

[a]TS incubated at 25°C with ^{14}C-PTC in 50 mM MES buffer, pH 6.0. [b]Expressed as percent activity of enzyme not treated with MMTS or PTC. [c]Calculated from CPM and specific activity of ^{14}C-PTC. [d]Enzyme pre-incubated with excess MMTS at 25°C before adding PTC.

TABLE II
Effect of HN_2OH and 2-mercaptoethanol on PTC binding

Step	Activity (%)		PTC/TS	
	20 min	4 h	20 min	4 h
1. (after PTC)[a]	5	0	2.2	4.5
2. (after NH_2OH)[b]	24	15	0.9	2.2
3. (after 2-ME)[c]	32	20	0.8	2.0

[a]TS was treated with ^{14}C-PTC at 25°C in 50 mM MES buffer, pH 6.0 for the times shown, then excess reagents were removed by chromatography on Sephadex G-25 with potassium phosphate buffer, pH 7.5. [b]PTC-treated TS from step 1 was treated at 25°C with 0.67 M NH_2OH at pH 7.5 for 4 h, then desalted by Sephadex G-25 chromatography. [c]TS from step 2 was dialyzed for 24 h in 20 mM potassium phosphate buffer, pH 6.8 containing 10 mM 2-mercaptoethanol.

been for 20 minutes with approximately 2 PTCs incorporated or for 4 h with 4-5 PTCs incorporated (Table II). Further dialysis against buffer containing 10 mM 2-mercaptoethanol resulted in an additional modest increase in catalytic activity with no further release of PTC.

When 18 μM thymidylate synthase was treated with 5 mM PTC in the presence of 100 mM ^{14}C-glycine ethyl ester (GEE), only 0.07 and 0.6 mol GEE were incorporated per mol of enzyme after 20 min and 4 h, respectively. However, 1.34 and 4.6 mol GEE were incorporated per mol of enzyme in the presence of 4 M urea for 4 h and 8 M urea for 12 h, respectively. These results indicate that the inactivation of TS is not accompanied by nucleophile incorporation. The GEE incorporation in the presence of urea indicates that PTC can act as a typical carbodiimide by activating carboxyl groups exposed by denaturation. Apparently, carboxyl groups in the native enzyme do not react with PTC and are not responsible for PTC incorporation or loss of activity.

Attempts to isolate ^{14}C-PTC-labeled cyanogen bromide or tryptic peptide fragments by reverse phase HPLC were unsuccessful and led to loss of radioactivity. No conditions for the preparation and purification of peptide fragments were found that resulted in retention of radioactivity.

DISCUSSION

All three carbodiimides used in this study inhibited thymidylate synthase from *Lactobacillus casei*, but the rate of inactivation varied widely. The rate of inactivation by PTC was 100-fold greater than that with EDC. Although this observation is consistent with earlier studies which showed that the rate of inactivation of thymidylate synthase by aromatic disulfides was greater than that with aliphatic disulfides,[7] the different degree of protection afforded by dUMP suggests that PTC and EDC may react with different sites on the enzyme. The greater rate of inactivation and the greater degree of protection suggests that PTC reacts at the active site.

However, the data reported here seem inconsistent with the usual reaction of carbodiimides with carboxyl groups to form *O*-acylisoureas, which then react with nucleophiles to form stable amides. Added nucleophiles had no effect on the rate of inactivation by PTC, nor was an appreciable amount of ^{14}C-glycine ethyl ester incorporated into the protein. An intramolecular amide formed from a PTC-activated

carboxyl group and a nucleophilic amino acid side chain, as reported by Tonner-Webb and Taylor,[14] also seems unlikely since labeled PTC was incorporated into the protein.

Amino acid side chains other than carboxyl groups known to react with carbodiimides are the cysteine sulfhydryl group,[12] the phenolic group of tyrosine,[13] and the hydroxyl group of serine.[15] The data presented that, (1) dUMP protects TS from inactivation by PTC, (2) reaction with PTC reduced the number of titratable enzyme sulfhydryl groups, and (3) prior blocking of sulfhydryl groups with MMTS protected the enzyme from inactivation by PTC, together with the known participation of a sulfhydryl group in the catalytic mechanism of TS, suggest that PTC reacts with cysteine residues to inactivate TS.

Acknowledgement

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